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TITLE: GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer

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# Title
GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer

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## Abstract
The present study examined the mechanistic basis for selenium upregulation of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) and the effect of GKLF overexpression on the growth of prostate cancer cells. The studies were conducted in the androgen-responsive LNCaP and the androgen-unresponsive, AR-null PC-3 cells. We found that selenium upregulates GKLF transcript through distinct mechanisms in the two cell types: a decrease in mRNA degradation in LNCaP and an increase in GKLF transcription in PC-3. Transfection of GKLF in PC-3 cells inhibited DNA synthesis and induced apoptosis. In contrast, LNCaP cells responded to GKLF transfection by increasing the level of androgen receptor (AR), and the effect of which predominated, leading to a modest stimulation of cell growth. We also found that selenium significantly decreases the expression of AR in LNCaP cells. Exogenous expression of AR greatly attenuated the growth suppressive activity of selenium, although the GKLF level was markedly induced after the transfection. Therefore, compared to the induction of GKLF, the disruption of AR signaling is probably more important for selenium action and more relevant to selenium chemoprevention of prostate cancer, since the vast majority of prostate cancers, including those refractory to hormone therapy, express a functional AR.

## Subject Terms
Selenium, cancer chemoprevention, prostate cancer, GKLF transcription factor
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A. INTRODUCTION:

A major goal of this project is to investigate the role of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) in contributing to the molecular effects of selenium in cancer chemoprevention. The reasons for focusing on GKLF are as follows. (a) The consensus element for GKLF is present at the highest frequency in the promoters of a cluster of early selenium-responsive genes. (b) The DNA-binding activity and expression level of GKLF are markedly induced by selenium treatment, and the induction could be detected as early as 1 hr after exposure to selenium. As an immediate target, it could modulate the transcription of a wide spectrum of genes and thus serve as a key trigger of selenium action. (c) Enforced expression of GKLF results in DNA synthesis inhibition in fibroblasts, cell growth arrest in vascular smooth muscle cells, and G1/S block and apoptosis in colon cancer cells (1-4). These effects are consistent with that of selenium. (d) GKLF expression is downregulated in colon and prostate cancers compared to normal tissues (5-7), thus suggesting a potential tumor suppressor function of GKLF. Conversely, the induction of GKLF expression and activity by selenium in prostate cancer cells may represent a rejuvenation of this role. During this first year of funding period, we examined the mechanistic basis for GKLF upregulation by selenium and the effect of GKLF overexpression on the growth of prostate cancer cells.

B. BODY:

Results for Task 1 (To study the mechanistic basis for GKLF upregulation by methylseleninic acid (MSA)):

MSA induces GKLF mRNA level. Fig. 1 shows MSA upregulation of the GKLF transcript, as determined by real-time RT-PCR, during a 6 or 16 hr period in LNCaP and PC-3 cells. The increase in GKLF transcript occurred very quickly. There was about a 2-fold induction in the first two hours after treatment with 10 μM MSA in both cell lines. In LNCaP cells, the induction by MSA reached the maximum at this time point. In contrast, the magnitude of induction rose to ~3-fold at 3 hr in PC-3 cells, and remained at this level upon longer exposure.

Fig. 1. Effect of MSA on AR mRNA expression as determined by real time RT-PCR analysis. The results are expressed as % of control; bars represent SEM. With the exception of the 1 hr data point in LNCaP cells, the remaining data points are statistically different (P < 0.01) from the untreated control.
**MSA does not change the activity of a 1 kb proximal GKLF promoter.** In order to study the effect of MSA on GKLF promoter activity, a luciferase reporter gene construct containing about 1 kb fragment of the proximal promoter region of the GKLF gene (kindly provided by Dr. Vincent W. Yang at the Emory University) was transiently transfected into LNCaP and PC-3 cells. The luciferase reporter assay was carried out at 1 hr, 2 hr, 3 hr, 6 hr, or 16 hr after treatment with 10 μM MSA. As can be seen in Fig. 2, MSA treatment did not result in any significant change in the activity of this 1 kb GKLF promoter. The data indicate that either MSA induces GKLF mRNA through increasing its mRNA stability, or the *cis* element(s) mediating MSA effect is not present in this 1 kb promoter region of the GKLF gene.

![Fig. 2](image)

**Fig. 2.** Effect of MSA on GKLF-promoter activity in LNCaP (A) and PC-3 cells (B). The results are expressed as % of control; bars represent SEM. None of the data points is statistically different (P < 0.01) from the untreated control.

**MSA increases the stability of GKLF mRNA in LNCaP cells.** We next performed an mRNA stability assay under the condition in which new RNA synthesis was blocked. Actinomycin D, an RNA synthesis inhibitor, was added to the culture at the time of MSA treatment, and GKLF mRNA levels were followed in a 6-hr time course by real-time RT-PCR analysis. Since actinomycin D could be cytotoxic, we also monitored cell growth for a duration of up to 8 hr and did not observe cell death or significant growth inhibition during this period. Our results showed that treatment with MSA increased the stability of GKLF mRNA in LNCaP cells, whereas no effect was observed in PC-3 cells (Fig. 3). Therefore, MSA could upregulate GKLF expression through distinct mechanisms in the two cell lines: a decrease in mRNA degradation in LNCaP cells and an increase in GKLF transcription in PC-3 cells.

![Fig. 3](image)

**Fig. 3.** Effect of MSA on GKLF mRNA stability in LNCaP (A) and PC-3 cells (B). The results are expressed as % of control; bars represent SEM.
Results for Task 2 (To determine the effect of GKLF overexpression on the growth of prostate cancer cells and the expression of potential GKLF-targeted genes):

**Overexpression of GKLF does not inhibit the growth of LNCaP cells.** In order to study the role of GKLF in regulating the growth of prostate cancer cells, we transiently transfected LNCaP cells with a GKLF expression construct, pcDNA3.1/His B-GKLF (kindly provided by Dr. Anil K. Rustgi at the University of Pennsylvania), and a membrane-GFP-encoding plasmid. The purpose of the green fluorescent protein (GFP) was to enable us to select for the subset of positively transfected cells. The pcDNA3.1/His B vector contains the gene encoding the Xpress epitope tag. None of the commercially available GKLF antibodies gave us specific signal on Western blots. In order to circumvent this problem, we used the pcDNA3.1 vector as a mock control in this study. The gene encoding the Xpress epitope tag is not included in the pcDNA3.1 vector. Therefore, we could use an antibody against the Xpress epitope tag to detect the expression of the transfected GKLF.

At 40 hr after transfection, one third of the cells were lysed and subjected to Western analysis with an anti-Xpress antibody, one third labeled with BrdU and analyzed by flow cytometry, and the remaining assessed for DNA fragmentation by using the Cell Death Detection ELISA PLUS kit (Roche). The BrdU data were analyzed by gating just the GFP-positive cells (i.e., positively transfected cells). As shown in Fig. 4A, GKLF overexpression did not slow down cell cycle progression in LNCaP cells. In fact, DNA synthesis was slightly induced by GKLF overexpression, although the induction was not statistically significant. Interestingly, we also observed a modest but statistically significant decrease of apoptotic cell death by GKLF overexpression (Fig. 4B). To confirm the functionality of the transfected GKLF protein, we cotransfected LNCaP cells with pcDNA3.1/His B-GKLF and a cyclin D1 promoter-luciferase construct, which contains the response element mediating GKLF-repression of cyclin D1. As expected, the activity of the cyclin D1 promoter in the GKLF-transfectants was reduced to ~40% of that in the mock-transfectants (data not shown).

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**Fig. 4.** Effect of GKLF overexpression on DNA synthesis and apoptosis in LNCaP cells. Panel A, BrdU labeling analysis of GKLF- or pcDNA3.1 (mock)-transfected LNCaP cells. The results are expressed as % of BrdU-staining in the selected positively transfected cells. Panel B, ELISA of DNA fragmentation in GKLF- or mock-transfected LNCaP cells. *, statistically different (P<0.01) from mock-transfectants. Western blot confirmation of Xpress protein level is shown in the inset.
Overexpression of GKLF induces apoptosis in PC-3 cells. We performed similar experiment in PC-3 cells, and found that PC-3 and LNCaP cells responded differentially to GKLF overexpression. A reduction of DNA synthesis and a modest but statistically significant induction of apoptosis were observed in PC-3 cells after exogenous expression of GKLF (Fig. 5). The BrdU analysis will be repeated and statistical analysis performed in order to determine whether the difference between the GKLF-transfectants and the mock-transfectants is significant.

Fig. 5. Effect of GKLF overexpression on DNA synthesis and apoptosis in PC-3 cells. Panel A, BrdU labeling analysis of GKLF- or pcDNA3.1 (mock)-transfected PC3 cells. The results are expressed as % of BrdU-staining in the selected positively transfected cells. Panel B, ELISA of DNA fragmentation in GKLF- or mock-transfected PC-3 cells. *, statistically different (P<0.01) from mock-transfectants. Western blot confirmation of Xpress protein level is shown in the inset.

Why do LNCaP and PC-3 cells respond differentially to GKLF overexpression? LNCaP and PC-3 cells have many distinct genetic components. One fundamental difference between the two cell lines is their sensitivity to androgen stimulation of growth. LNCaP cells are androgen responsive, whereas PC-3 cells androgen unresponsive. LNCaP cells express a mutant but functional androgen receptor (AR), whereas PC-3 cells are AR-null. Androgen plays a key role in regulating prostate cell growth. Since the response of androgen is mediated by binding to the AR, we next examined the effect of GKLF overexpression on AR protein level by Western blot analysis. As shown in Fig. 6, AR protein level was greatly induced after GKLF transfection. Thus, the induction of AR and possibly androgen signaling might be a compensatory mechanism for LNCaP cells to maintain homeostasis after the introduction of the GKLF growth inhibitory factor. In contrast, no such compensatory pathway could be activated in the AR-null PC-3 cells. Therefore, we observed a suppression of DNA synthesis and an induction of apoptosis after GKLF transfection.

MSA represses AR expression in LNCaP cells. Our next step was to investigate the expression of AR protein in response to MSA by Western analysis. In contrast to GKLF overexpression, MSA downregulated AR protein level (Fig. 7). Within the first 3 hr of treatment
with 10 µM MSA, there was a 40% decrease in AR protein. The AR protein level continued to drop down to 10% or below with longer treatment with MSA (Fig. 7).

**Fig. 7.** A, Western analysis of AR level as a function of time of treatment with 10 µM MSA. B, quantitative determination of the Western data.

**Overexpression of AR attenuates MSA-mediated growth inhibition.** In order to delineate the role of low AR abundance in mediating MSA action, we investigated whether the growth inhibitory effect of MSA could be mitigated in AR-transfected cells. An AR expression vector or the mock plasmid was introduced into LNCaP cells with the co-transfection of a membrane-GFP-encoding construct. At 24 hr after treatment with 10 µM MSA, the cells were subjected to BrdU-labeling, and the data were analyzed by gating just the GFP-positive cells. As shown in Fig. 8, MSA inhibited DNA synthesis by a very modest 16% in the AR-transfectants, as opposed to 72% in the mock-transfectants. In other words, AR overexpression greatly weakened the growth suppressive activity of MSA. We also examined GKF mRNA level in the transfecteds by real-time RT-PCR, and found that the AR-transfectants expressed about 10-fold more GKF mRNA than the mock-transfectants (data not shown). Thus, the induction of GKF might also be a compensatory mechanism for cells to maintain homeostasis after the transfection of the AR. However, the fact that we observed a marked attenuation of MSA growth inhibition by AR overexpression indicates a predominant role of the AR signaling pathway in MSA action.

**C. KEY RESEARCH ACCOMPLISHMENTS:**

- In LNCaP cells, selenium upregulates GKF mRNA level through decreasing GKF mRNA degradation.
- In PC-3 cells, selenium upregulates GKF mRNA level through increasing GKF transcription. The cis element(s) mediating such effect of selenium is not present in the 1-kb proximal promoter region of the GKF gene.
Exogenous expression of GKLF prevents the androgen-responsive LNCaP cells from undergoing apoptosis.

Overexpression of GKLF inhibits DNA synthesis and induces apoptosis in the AR-null PC-3 cells.

In LNCaP cells, AR protein level is greatly induced after GKLF transfection. The induction of AR and possibly androgen signaling might be a compensatory mechanism for LNCaP cells to maintain homeostasis after the introduction of GKLF.

Selenium treatment results in a rapid and marked repression of AR expression in LNCaP cells.

Exogenous expression of AR significantly mitigates the growth suppressive activity of selenium in LNCaP cells.

GKLF mRNA level is greatly elevated in the AR-transfected LNCaP cells as compared to the mock-transfected control cells. The induction of GKLF might also be a compensatory mechanism for cells to maintain homeostasis after the transfection of the AR. However, the fact that we observed a marked attenuation of selenium growth inhibition by AR overexpression indicates a predominant role of the AR signaling pathway in selenium action.

D. REPORTABLE OUTCOMES:

- Funding received:
  NCI Howard Temin (K01) Career Development Award (Dong, PI) 04/05 – 03/10
  - Award per year: $120,000

- Employment received:
  Assistant Member, Dept. of Cancer Chemoprevention, Roswell Park Cancer Institute, Buffalo, NY 14263

E. CONCLUSIONS:

The results from the current study indicate a growth suppressive and pro-apoptotic function of GKLF in the AR-null PC-3 cells. However, the LNCaP cells respond to GKLF overexpression by an induction of AR, and the effect of which predominates, leading to a modest stimulation of cell growth. We also found that selenium is able to markedly suppress AR expression. Exogenous expression of AR greatly attenuates the growth inhibitory activity of selenium, although accompanied by a significant increase in GKLF level. The data suggest that the disruption of AR signaling is probably more important than the induction of GKLF signaling for selenium action.

Our microarray data showed that selenium modulates the expression of a multitude of genes and targets many different signaling pathways. Since the ultimate goal of our research is to unravel the molecular mechanism of selenium chemoprevention of prostate cancer, we should prioritize our study of the gene/pathway according to their importance to selenium action.
vast majority of prostate cancers express a functional AR. Although GKLF has growth suppressive activity in the AR-null cells, such activity might be overshadowed by AR signaling in AR-expressing cells.

Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the AR signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (8). The development of hormone refractory prostate cancer is not associated with loss of AR (9,10). Instead, the accumulation of several molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Amplification and/or overexpression of AR can hyper-sensitize cells to sub-physiological levels of androgens (11-14). A recent report by Chen et al. (11) claimed that increased AR expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent, and that AR antagonists may display agonistic activity in cells with elevated AR expression. In addition, AR gene mutations could result in a promiscuous receptor with a broad ligand-binding and trans-activation spectrum (15). A selenium intervention strategy aimed at diminishing the expression of AR could be helpful not only for reducing incident prostate cancer, but also for preventing relapses after endocrine therapy.

Based on the above observation and the critical role of AR signaling in prostate cancer growth, we would like to request a change on our future work. We would like to continue our GKLF study in the AR-null PC-3 cells, but shift our future research focus to selenium suppression of AR signaling in the androgen-responsive LNCaP cells. Two key questions will be addressed. First, is AR downregulation critical for selenium inhibition of prostate cancer cell growth? Second, How does selenium suppress androgen receptor signaling? The experimental design will be similar to the one outlined in Aims 1 & 2 in the proposal for the study of the GKLF gene. We would greatly appreciate your kind consideration of our request.

F. REFERENCES:


